REMARKS

Receipt is acknowledged of the captioned action, where the examiner rejected claims 1-47 for alleged non-enablement. The examiner also rejected claims 1-22, 24-31 and 41-47 for alleged indefiniteness. Additionally, the examiner maintained her anticipation rejection of claims 1, 7-11, 17-25 and 28-32 over U.S. patent No. 6,310,045, and of claims 23 and 32 over Hollenbaugh and Sturmhoefel, respectively.

Status of the Claims

In this amendment, claims 1, 2, 7, 12, 20, 23-26, 29, 32, 45 and 46 have been amended and claims 48-56 have been added. Support for these amendments and new claims, respectively, can be throughout the specification. Claims 22 and 31 have been cancelled. Upon entry of this amendment, claims 1-21m 23-30 and 32-56 are pending.

The present amendment does not necessitate a further search and generally accommodates the examiner's concerns made in the outstanding office action. More particularly, the claim amendments and new claims speak to the enablement and indefiniteness rejections, and focus on the B7 family of costimulatory factors and localized delivery of the claimed composition. These points were already raised by the examiner in the current action and therefore do not present new issues. Accordingly, applicants respectfully request entry of the amendment and reconsideration of the present rejections in light of the following remarks.

35 U.S.C. § 112, first paragraph

The examiner has acknowledged that "the specification is enabling for methods of reducing the growth of a solid neuroblastoma by intratumoral injection of a defective Herpes Simplex Virus (HSV) vector encoding a soluble B7-1-Ig fusion protein" (office action at 5). Still, the examiner maintained her rejection of claims 1-32, and has newly rejected claims 33-47, for alleged non-enablement. Specifically, the examiner asserted that, in "the absence of sufficient levels of peptide/MHC on the cell surface, the tumor cannot be targeted by activated T cells," and that "the level of peptide/MHC on the cell surface [the neuroblastoma] has been demonstrated to be ***sufficient*** to allow lysis of the cells" (page 3; emphasis added).

The examiner's assertions may reflect a misunderstanding of applicants' invention, however. That is, the claimed invention concerns an approach for "souping up" the immune system by expressing a costimulatory factor, irrespective of MHC class I expression. Thus, the fact that a tumor cell has low or no MHC class I expression has no particular relevance to the present context. In any event, tumor cells that are categorized as "class I negative," based on immunohistochemical results, in fact retain some HLA class I expression, as shown, for instance, by Sette *et al.*, *Immunogenetics,* 53:255 (2001) (Appendix A hereto), and CTL recognition of as few as 1 MHC/peptide complex can lead to tumor cell lysis.

Neuro2A cells are merely illustrative here, in that other tumor types than a neuroblastoma can be treated in accordance with the invention. Indeed, MHC class I-negative cell lines, such as TRAMP-C1 and C2 cells, still can engender an immune-mediated effect when induced with IFNγ. See Grossman *et al.*, *World J. Urol.* 19: 365 (1990). Therefore, it is unsound scientifically for the examiner to assert that a "sufficient" level of a peptide/MHC complex must be present to launch an effective immune response. Put another way, the low level or lack of MHC class I expression is not determinative of whether a T-cell response is activated.

The examiner also has urged that the specification is enabling for a defective HSV vector only (page 5). Applicants respectfully disagree. Informed by their specification, one of skill in the art would know how to make and use other vectors in this regard. Indeed, applicants themselves constructed a recombinant, oncolytic HSV vector, vHSV-B7Ig, which expressed B7-Ig. Treatment of Neuro2a subcutaneous tumors by administration of vHSV-B7Ig significantly inhibited tumor growth, compared to control (PBS plus 10% glycerol), and was more effective than vHSV (Appendix B).

The generality of vectors useful for the invention is a teaching by applicants that stands uncontroverted on the record, which includes data that underscore the suitability of two vector types in the invention. Accordingly, the examiner is unreasonable to assert that only defective HSV vectors are enabled.

In similar fashion, the examiner has cited *In re Wands* and asserted that the specification is enabling for a B7-1-Ig costimulatory factor alone (page 5). Again, applicants must respectfully disagree. The B7 family members have been known in the art at the time of

filing and based on the structural and functional homology among the B7 family members, one skilled in the art would know how to select other B7 family members for use in the instant invention. In this regard applicants would point to Carreno *et al.*, which describes the homology between B7-1 and B7-2, and other B7 family members. See Carreno *et al.*, *Annu. Rev. Immunol.* 20: 29 (2002), in Appendix C. For example, Carreno stated that "[i]nclusion in the B7 family of ligands is based upon the degree of homology, as well as evidence of costimulatory or inhibitory function in immune assays" (*id.* at 31). Therefore, the examiner's proffered basis is untenable for reading the scope of enablement so narrowly, limited as it is to B7-1.

In this vein, the examiner referred to a previous office action (paper No. 5), dated December 19, 2001, purportedly to support her assertion that "there is no evidence of record that other delivery methods would work" (office action at 8). Paper no. 5 detailed problems associated with *sustained expression* of a therapeutic gene. For example, the examiner cited Verma *et al.*, *Science.* 389: 239 (1997), for the notion that "most of the [gene therapy] approaches suffer from poor efficiency of delivery and ***transient expression*** of the gene" (page 6; emphasis added). The examiner also quoted Marshall, *Science*, 269: 1054 (1995), who highlighted "difficulties...in getting genes transferred efficiently to target cells and [in] ***getting them expressed*** remain a nagging problem in the entire field" (*id.*; emphasis added).

Thus, a major concern of the examiner with respect to enablement relates to whether expression of the gene will be more than transient. In this regard, it is important to distinguish the situation in which gene therapy is used to correct a genetic disorder from gene therapy used according to the instant invention. Sustained expression of the costimulatory factor in the present invention is not essential and, in fact, is not desired. Transient gene expression is sufficient to trigger T-cell stimulatory activity.

As to whether all delivery methods would be equally effective in delivering the expressible sequence, in the interest of expediting prosecution, applicants are amending claims 1, 7, 24, 25, 45 and 46 to recite "localized" delivery. Support for this amendment can be found on page 6 of the specification, lines 21-32, which describes the advantages of localized delivery of soluble co-stimulatory factors over systemic administration.

35 U.S.C. § 112, second paragraph

The examiner rejected claims 1-22, 24-31 and 41-47 as the term "tumor-related cells" is allegedly indefinite. Although one of skill in the art would know what is meant by "tumor-related cells," and the rejected claims are clear on their face, claims 1, 2, 20, 24 and 29 have been amended to recite "cells in the immediate area of the tumor." Support for this amendment can be found on page 3, lines 1-2 of the specification.

35 U.S.C. § 102(e)

The examiner rejected claims 1, 7-11, 17-25 and 28-32 as being anticipated by Barber *et al*, asserting that "the claims as written simply recite a 'soluble co-stimulatory factor'" and that IL-2 "was recognized by the art as a co-stimulatory factor" (office action at 10). As discussed above, claims 1, 23, 24 and 32 have been amended to recite a "co-stimulatory factor in the B7 family." We trust that these amendments will alleviate the examiner's concerns.

35 U.S.C. § 112 § 102(b)

The examiner maintained her rejection of claims 23 and 32 as being anticipated by Hollengbaugh *et al*. Specifically, the examiner asserted that "applicant's claims...do not specifically recite a 'gene therapy vector'" and that "applicant has not presented any evidence that the vector taught by Hollenbaugh et al. is structurally different from the applicant claimed composition (office action at 11). Applicants respectfully disagree. Hollenbaugh uses soluble gp39, which is not a member of the B7 family. Additionally, gp39 is a type II membrane protein (as opposed to B7) and, therefore, was fused to a CD8 extracellular domain. Nevertheless, applicants amended claims 23 and 32 to recite "a gene therapy vector" (claim 23) and "a soluble co-stimulatory factor in the B7 family" (claims 23 and 32).

35 U.S.C. § 112 § 102(a)

The examiner maintained her rejection of claims 23 and 32 as being anticipated by Sturmhoefel, stating that "[t]he applicant's claims...do not specifically recite a 'gene therapy vector'" (office action at 12). Applicants trust that the claim amendments discussed above obviate this rejection.

In summary, the examiner seems to have unduly relied on applicants' working examples to determine what is enabled by the specification. In particular, the examiner asserted that the specification is *only* enabling for reducing the growth of a solid neuroblastoma, by intratumoral injection of a defective HSV vector encoding a soluble B7-1-Ig fusion protein. This position is not sound since working examples are actually not required. See M.P.E.P. § 2164.02. Thus, applicants' working examples should not be the touchstone for the enabling quality of their specification.

Furthermore, the level of one of ordinary skill in the art is important and is not being considered by the examiner. Indeed, a skilled artisan would be able to select a vector suitable for use in the present invention and a costimulatory factor in the B7 family based on the teachings in the specification. Moreover, given the structural and functional similarities among the B7 family members, and B7-1 and B7-2 in particular, the quantity of experimentation required to select a B7 costimulatory factor would not be undue.

Finally, the examiner's understanding of the present invention is flawed. The examiner cited references that describe the problems associated with sustained expression of a therapeutic gene and asserted that a sufficient level of peptide/MHC is required for cell lysis. However, the examiner fails to appreciate that transient gene expression is enough to induce an immune response and that cell lines considered to be MHC class I negative can be targeted by T cells.

Therefore, in view of these remarks, applicants respectfully request that the rejections be withdrawn.

CONCLUSION

Applicants submit that this application is in condition for allowance, and they solicit an early indication to that effect. Should the examiner believe that further discussion of any remaining issues would advance the prosecution, a telephone call to the undersigned, at the telephone number listed below, is courteously invited.

Respectfully submitted,

Date: November 18, 2002

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VERSION WITH MARKINGS TO SHOW CHANGES MADE

1. (Amended) A gene therapy method of activating or enhancing a T-cell response in a patient with a tumor, comprising administering to said patient, via localized delivery, a pharmaceutical composition comprising: (A) an expressible nucleotide sequence for a soluble costimulatory factor in the B7 family and (B) a vector, such that (i) said factor is expressed by **[the]** tumor cells or **[the]** cells in the immediate area of the tumor **[tumor-related cells]**, and (ii) said T-cell response thereby is activated or enhanced against said tumor.

2. (Amended) The method according to claim 1, wherein said vector is targeted to tumor cells or cells in the immediate area of the tumor **[tumor-related cells]**.

7. (Amended) The method of claim 1, wherein said localized delivery **[administrating]** comprises introducing said composition directly into said tumor or a local area of said tumor.

12. (Amended) The method according to claim 1, wherein said factor is selected from the group consisting of B7-1**[,]** and B7-2 **[B7-3, CD40, CD40 ligand, CD72, CD24, LFA-3, ICAM-1, CD70, CD2, CD48, 4-1BB, 4-1BB ligand, and LIGHT]**.

20. (Amended) The method of claim 1, wherein said tumor cells or cells in the immediate area of the tumor **[tumor-related cells]** are selected from the group consisting of melanoma cells, pancreatic cancer cells, prostate carcinoma cells, head and neck cancer cells, breast cancer cells, lung cancer cells, colon cancer cells, ovarian cancer cells, renal cancer cells, neuroblastomas, squamous cell carcinomas, hepatoma cells, and mesothelioma and epidermoid carcinoma cells.

23. (Amended) A pharmaceutical composition comprising (A) a gene therapy vector that contains a gene encoding a soluble costimulatory factor in the B7 family and (B) a pharmaceutically compatible carrier.

24. (Amended) A gene-therapy method of activating or enhancing a T-cell response in a patient with a tumor, comprising administering to said patient, via localized delivery, a pharmaceutical composition comprising: an expressible nucleotide sequence for a soluble costimulatory factor in the B7 family, such that (i) said factor is expressed by **[the]**

tumor cells or cells in the immediate area of the tumor **[tumor-related cells]**, and (ii) said T-cell response thereby is activated or enhanced against said tumor.

25. (Amended) The method according to claim 24, wherein said localized delivery **[administrating]** comprises introducing said composition directly into said tumor or local area of said tumor.

26. (Amended) The method according to claim 24, wherein said factor is selected form the group consisting of B7-1**[,]** and B7-2 **[B7-3, CD40, CD40 ligand, CD72, CD24, LFA-3, ICAM-1, CD70, CD2, CD48, 4-1BB, 4-1BB ligand, and LIGHT]**.

29. (Amended) The method of claim 24, wherein said tumor cells or cells in the immediate area of the tumor **[tumor-related cells]** are selected from the group consisting of melanoma cells, pancreatic cancer cells, prostate carcinoma cells, head and neck cancer cells, breast cancer cells, lung cancer cells, colon cancer cells, ovarian cancer cells, renal cancer cells, neuroblastomas, squamous cell carcinomas, hepatoma cells and mesothelioma and epidermoid carcinoma cells.

32. (Amended) A pharmaceutical composition comprising (A) a gene encoding a soluble costimulatory factor in the B7 family and (B) a pharmaceutically compatible carrier.

45. (Amended) The method according to claim 42, wherein said localized delivery **[administrating]** comprises introducing said composition directly into said tumor or a local area of said tumor.

46. (Amended) The method according to claim 45, wherein said localized delivery **[administering]** comprises directly injecting said nucleotide sequence or directly injecting said nucleotide sequence conjugated to a liposome carrier.

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A

ORIGINAL PAPER

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HLA expression in cancer: implications for T cell-based immunotherapy

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Abstract HLA class I expression is altered in a significant fraction of the tumor types reviewed here, reflecting either immune pressure or, simply, the accumulation of pathological changes and alterations. However, in all tumor types analyzed, a majority of the tumors express HLA class I, with a general tendency for the more severe alterations to be found in later-stage and less differentiated tumors. These results are encouraging for the development of specific immunotherapies, especially considering that (1) the relatively low sensitivity of immunohistochemical techniques might underestimate HLA expression in tumors, (2) class I expression can be induced in tumor cells as a result of local inflammation and lymphokine release, and (3) class I-negative cells would be predicted to be sensitive to lysis by natural killer cells.

Keywords HLA · Class I · Tumor · Epitope · Immunotherapy

Disease progression in cancer and infectious disease

A dynamic interaction exists between host and disease, both in the cancer and infectious-disease settings. In the latter, pathogens evolve during disease. For example, the viral sequences that predominate early in HIV infection differ from those associated with AIDS and the terminal disease stages. Pathogen forms that are effective in establishing infection are believed to differ from those most effective in terms of replication.

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The pathological process by which an individual succumbs to a neoplastic disease is a complex phenomenon. During the course of disease, many changes occur within the cancer cells. The tumor accumulates alterations which are in part related to a seemingly random, vicious circle of dysfunctional regulation of growth and differentiation, but are also related, to some degree, to maximizing its growth potential, and to escaping drug treatment and/or the body's immunosurveillance.

An in-depth discussion and review of these topics is beyond the scope of the present article. Here, we emphasize that neoplastic disease is a complex and dynamic process, which results in the accumulation of several different biochemical alterations of cancer cells as a function of disease progression. It also results in significant levels of intra- and intercancer heterogeneity, particularly in the late metastatic stage. This situation ultimately translates into a significant heterogeneity of approaches and efficacies of drugs targeting neoplastic diseases.

All too familiar examples of cellular alterations affecting treatment outcomes include the outgrowth of radiation- or chemotherapy-resistant tumors during the course of therapy. Significant heterogeneity of responses also appears to be associated with other newer approaches to cancer therapy, including anti-angiogenesis drugs, passive antibody immunotherapy, and active T cell-based immunotherapy. These examples are analogous to the unfortunate emergence of drug-resistant viral strains as a result of aggressive chemotherapy of chronic hepatitis B virus and HIV infection, and the current resurgence of drug-resistant tuberculosis and malaria.

The interplay between disease and the immune system

One of the main factors contributing to the dynamic interplay between the host and disease is the immune

response mounted against the pathogen. In infectious diseases, the relationship between immune responses and the appearance of escape mutants has been well characterized in a variety of disease settings, including HIV, SIV, hepatitis C virus, leishmaniasis, and malaria. Immune responses can, in certain conditions, control these infections. Several different animal model systems and prospective studies of natural infection in humans suggest that immune responses against the pathogen of interest can lead to prevention and/or therapeutic elimination of disease. A common motif from this work is the requirement for a multispecific T-cell response, and the lower effectiveness of narrowly focused responses. These observations have spurred significant enthusiasm, and guide the development of various specific immunotherapies and vaccines.

In the cancer setting, there are several indications that immune responses can impact neoplastic growth. First, the studies in many different animal models have demonstrated that antitumor T cells, restricted usually by MHC class I, can prevent or treat tumors. Second, encouraging results have been obtained from immunotherapy trials. Third, observations made in the course of natural disease have correlated the type and composition of T-cell infiltrate within tumors with positive clinical outcomes (Coulie et al. 1999). The presence of monospecific cytotoxic lymphocytes (CTLs) was also correlated with control of tumor growth, until antigen loss emerged (Marchand et al. 1999; Riker et al. 1999). Similarly, loss of β_2-microglobulin was detected in 5/13 lines established from melanoma patients after receiving immunotherapy at the National Cancer Institutes (Restifo et al. 1996). Finally, HLA class I is frequently altered in various tumor types, leading to the speculation that this phenomenon might reflect immune pressure exerted on the tumor by means of class I-restricted CTLs. Several studies have proposed that the extent and degree of alteration in HLA class I expression not only reflect past immune pressures, but might also have prognostic value (van Duinen et al. 1988), even though this issue is still controversial. Taken together, these observations provide a rationale for immunotherapy of cancer, and at the same time suggest that effective strategies need to account for the complex series of pathological changes associated with neoplastic disease. In the following sections we will focus on one such change, namely the decrease in HLA expression in tumors, especially as it relates to antigen-specific T-cell immunotherapy.

Assaying HLA expression in tumor cells and lines

Various approaches have been utilized over the years to assay for expression of HLA class I on tumor cells. By far the most widely utilized method is immunohistochemical staining of formalin-fixed or frozen tumor sections. The lack of standardized protocols that can be applied consistently by different investigators has rendered it difficult to compare results obtained in different studies.

Analysis using fluorescence-activated cell sorting (FACS) appears to be the most accurate and reliable method. It is routinely performed utilizing well-standardized protocols, and allows one to simultaneously quantitate expression on normal versus tumor cells, separated by the use of staining for specific tumor markers. Analysis using FACS is of higher sensitivity than immunohistochemistry, and several recent publications have emphasized that staining of formalin sections can lead to serious underestimation of class I expression, both in terms of levels of expression and numbers of cells positive for expression. Recent studies indicate that some tumors, which would have been scored as class I negative by immunohistochemistry, do express low levels of class I molecules when analyzed by FACS (Diederichsen et al. 1998; Koopman et al. 2000; Tait 2000).

In terms of reagents, anti-class I, pan-reactive antibodies, such as W6/32, have been widely utilized. This approach although generally useful cannot detect allele-specific losses or decreases. To overcome this limitation, allele-specific antibodies are also routinely utilized. The use of allele-specific reagents is limited by the availability of well-characterized specific antibodies, and by their consistent use by different laboratories. To overcome these limitations, a section of the 13th International HLA Workshop, chaired by Dr. S. Ferrone, will specifically address the issues of quality control of protocols and reagents utilized for tumor cell staining.

Another hurdle to standardization and interpretation of immunohistochemistry assay are the classification and definition of what represents a significant loss/decrease in expression. A large fraction of cells within a given tumor can exhibit total loss, while the remaining cell population might exhibit normal expression. In most cases, this might be classified as an instance of total loss, while in functional terms, the tumor would still be subject, in large part, to direct attack from the immune response. Furthermore, in the case of heterogeneous expression, both normally expressing and defective cells are predicted to be susceptible to immune destruction based on "bystander effects" (see below). Related to these issues, a study from Esteban and co-workers (1996) investigated a total of 60 tumors of laryngeal origin.

Alternative assays are also available but are utilized less frequently. These involve assaying for the presence of specific mRNAs as well as gel electrophoresis of specific HLA products. Nucleic acid-based assays have the potential for higher sensitivity. Klein and co-workers (1996), for example, noted that HLA class I expression is not lost from colorectal cancers, despite their scoring for negative expression at the level of detection by antibody staining.

The three main types of alterations in HLA expression in tumors and their functional significance

The level and pattern of expression of HLA class I antigens in tumors has been studied in many different tumor types. For the reasons mentioned in the preceding paragraph, reaching a consensus interpretation of studies performed from different investigators is sometimes difficult. Nevertheless, alterations have been reported in all types of tumor studied and, in general, appear to impact a sizable fraction of the individual tumors studied. The molecular mechanisms generating HLA class I alterations have been demonstrated to be quite heterogeneous. They include alterations in the TAP/processing pathways, mutations of β_2-microglobulin and specific HLA heavy chains, alterations in the regulatory elements presiding over class I expression, and even loss of entire chromosome sections. Reviewing these molecular mechanisms is beyond the scope of this paper, but several excellent reviews on this topic are available (Garrido et al. 1993; Kaklamanis et al. 1992). Here we will distinguish three main types of alteration (complete loss, allele-specific loss, and decreased expression). The functional significance of each alteration will be discussed separately in the following sections.

Complete loss of HLA expression

Complete loss of HLA expression can result from a variety of different molecular mechanism (for reviews see Algarra et al. 2000; Browning et al. 1996; Ferrone and Marincola 1995; Garrido et al. 1993; Tait 2000). In functional terms, this type of alteration has several important implications. First, complete absence of class I expression renders the tumor cells extraordinarily sensitive to lysis by natural killer (NK) cells (Liunggren and Karre 1985; Maio et al. 1991; Ohnmacht and Marincola 2000; Schrier and Peltenburg 1993).

A possible caveat to consider in this regard is that recent studies have highlighted how the expression of HLA-G in tumor cells might inhibit NK cell activity (Paul et al. 1998). The complementary interplay between loss of HLA expression and gain in NK sensitivity is exemplified by the classic studies of Coulie and co-workers (1999) which described the evolution of a patient's immune response over the course of several years. Because of increased sensitivity to NK lysis, therapeutic approaches leading to stimulation of innate immunity in general and NK activity in particular are predicted to be of special significance. An example of such an approach is the induction of large amounts of dendritic cells by various hematopoietic growth factors, such as Flt3 ligand and ProGP. The rationale for this approach resides in the well-known fact that dendritic cells produce large amounts of interleukin (IL)-12, one of the most potent stimulators known for innate immunity, and NK activity in particular. Interesting in light of this is that Flt3 ligand treatment results in transient tumor regression of a mouse class I-negative prostate cancer model (Ciavarra et al. 2000). In this context, specific antitumor vaccines are predicted to synergize well with these types of hematopoietic growth factors.

Second, complete loss of HLA frequently occurs only in a subset of the tumor cells. A large fraction of the cells within a given tumor can exhibit total loss, while the remaining cell population might exhibit normal expression. In most cases this would be classified as an instance of total loss, while in functional terms, the tumor would still be largely subject to direct attack from the immune response. In the case of heterogeneous HLA expression, both normally expressing and defective cells are predicted to be susceptible to immune destruction based on bystander effects, as demonstrated, for example, in the studies of Rosendahl and co-workers (1998) investigating the in vivo mechanism of action of antibody-targeted superantigens. In the worst-case scenario, the fraction of tumor cells that do not express HLA would be spared from NK cell lysis and from bystander effects. Even in this case, destruction of the HLA-expressing fraction of the tumor by immune-mediated mechanisms would still have potentially dramatic effects on survival times and quality of life, leading to registrable products. Furthermore, van Driel and co-workers (1996) showed that HLA class I-negative tumors injected in nude mice will change in this NK environment into HLA class I-positive tumors. A similar dynamic mechanism may also affect immunotherapy, despite the fact that at the moment the expression is checked, sufficient HLA molecules may be available for effective therapy. Therefore, caution should be exercised in predicting a priori, the responsiveness of a given tumor to immunotherapy.

Allele-specific loss

One of the most common types of alteration in class I molecules is represented by the selective loss of certain alleles. This type of alteration might go undetected or at least unrecognized if the different HLA molecules potentially expressed in the cell are not measured independently. Fortunately, many studies have been able to incorporate enough allele-specific antibodies in their protocols to allow measurement of this important alteration. Allele-specific alterations might reflect tumor adaptation to immune pressure, exerted by an immunodominant response, restricted by a single HLA restriction element. This type of alteration allows the tumor to retain class I expression and thus escape NK cell recognition. If this hypothesis were to be proven experimentally, it would constitute important support for the concept of immunotherapy of cancer in humans, by providing data indicating that

the immune system has the capacity to control the tumor.

A practical solution to overcoming the potential hurdle of allele-specific loss relies on the induction of multispecific responses. Just as the inclusion of multiple tumor-associated antigens in a vaccine construct guards against mutations leading to loss of any specific tumor antigen, simultaneously targeting multiple HLA specificities with a unique, multiepitopic, multispecific construct should allow for the prevention of tumor escape by allele-specific losses.

Decrease in expression (allele specific or multiallele)

The biological significance of decreased HLA expression in possible tumor escape from immune recognition is presently unclear. Three main lines of experimental observation must be considered. First, CTL recognition of as few as one MHC/peptide complex has been demonstrated to be sufficient to lead to tumor cell lysis. Second, expression of HLA is commonly observed to be upregulated by interferon (IFN)-γ, commonly secreted by effector CTLs. Third, decreased levels of HLA class I expression also render cells more susceptible to NK lysis.

The exquisite sensitivity of effector CTLs has been known for some time (Brower et al. 1994; Christinck et al. 1991; Sykulev et al. 1996). As mentioned above, even a single peptide/MHC complex can result in tumor cells lysis and release of antitumor lymphokines.

With regard to IFN-γ, Torres and co-workers (1996) noted that HLA molecules are induced by IFN-γ in pancreatic cancer, unless a total loss of haplotype has occurred. Similarly, Rees and Mian (1999) noted that decreased expression of *HLA-A*, *-B*, and *-C* genes (allelic-specific loss) can be restored, at least partially, by cytokines such as IFN-γ. In an independent study, IFN-γ treatment was noted to result in upregulation of class I molecules in the majority of the cases studied (Browning et al. 1996), and Kaklamanis and co-workers (1995) suggested that adjuvant immunotherapy with IFN-γ may be of benefit in the case of HLA class I-negative tumors. IFN-γ production is induced and self-amplified by local inflammation/immunization (Halloran et al. 1992), resulting in large increases in MHC expressions even in sites distant from the inflammatory site. However, Kooy and co-workers (1999) recently reported that fairly high doses of recombinant (r)IFN-γ failed to upregulate HLA class I molecules in basal cell carcinomas. Interestingly, in their case, the effect appeared to depend on the inhibitory effect of IL-10, a lymphokine whose inhibitory effects are antagonized by IL-12, a lymphokine abundantly produced by dendritic cells. Of relevance to immunotherapy, class I expression can also be induced in vivo by both IFN-α and IFN-β (Halloran et al. 1992; Pestka et al. 1987).

Finally, several studies have demonstrated that decreased expression can render tumor cells more susceptible to NK lysis (Liunggren and Karre 1985; Maio et al. 1991; Ohnmacht and Marincola 2000; Schrier and Peltenburg 1993). If decreases in HLA expression benefit the tumor because they facilitate CTL escape but render the tumor susceptible to NK lysis, then the minimal level of HLA expression that would allow for resistance to NK activity would be selected for (Garrido et al. 1997), implying that IFN-γ treatment or infiltration by high-affinity T cells would render the tumor CTL sensitive.

Frequency of alterations in HLA expression

Several different studies have addressed the type and frequency of alterations in class I expression in general (Algarra et al. 2000). Rees and Mian (1999) estimated allelic-specific loss to occur overall in 3–20% of tumors, and allelic deletion to occur in 15–50% of tumors. Because each cell carries two separate sets of class I genes, each carrying one HLA-A and one HLA-B locus, the actual frequency of losses for any specific allele could be as little as one-quarter of the overall frequency. They also noted that, in general, a gradient of expression exists between normal cells, primary tumors, and tumor metastasis. In a different study, Natali and co-workers (1984) investigated solid tumors for total HLA expression using the W6/32 antibody, and for allele-specific expression of the A2 antigen, as evaluated by BB7.2 staining. Tumor samples were derived from either primary cancers or metastasis, for 13 different tumor types, and scored as negative if less than 20%, reduced if in the 30–80% range, or normal. All tumors, both primary and metastatic, were HLA positive with W6/32. For A2 expression, a reduction was noted in 16.1% of the cases, and A2 was scored as undetectable in 39.4% of the cases. Garrido and co-workers (1993) emphasize that HLA changes appear to occur at a particular step in the progression from benign to most aggressive. Jimenez and co-workers (2000) have analyzed 118 different tumors (68 colorectal, 34 laryngeal, and 16 melanomas). The frequencies reported for total loss of HLA expression were 11% for colon, 18% for melanoma, and 13% for larynx.

We shall now briefly review some of the data available for tumor types of major interest to us, in the context of some of the main types of alteration (complete loss, allele-specific loss, decreased expression, and allele-specific decreased expression). Particular emphasis will be placed on the correlation between these alterations, tumor stage, and disease progression. Because our ultimate goal is the development of specific immunotherapy, we shall also calculate/determine in each case, the fraction of tumors derived from individuals positive for a given HLA-A specificity (for example A2) which still express detectable class I. In

particular, close attention must be paid to distinguishing between the overall fraction of tumors presenting alterations of *any type* versus the fraction of tumors presenting alteration in a *given HLA type* targeted by immunotherapy. Many alterations are allele specific and four different HLA-A or -B class I molecules are usually encoded in a given individual (one HLA-A and HLA-B in each chromosome). As a result, the fraction of tumors showing any alterations is usually much greater than the fraction of tumors showing alteration in a specific HLA molecule.

For example, Koopman and co-workers (2000) used flow cytometry and molecular analyses to concurrently assess allele-specific HLA phenotypes and genotypes in subpopulations from 30 freshly isolated cervical tumors. Because the actual data were presented, the fraction of tumors from A2 individuals retaining A2 expression can also be evaluated. Of 16 tumors derived from A2-positive individuals, 8 retained expression (7 had normal expression and 1 reduced). Thus, the fraction of A2 individuals predicted to be amenable to A2-specific immunotherapy is 50% for this late-stage population.

Breast cancer

van Belzen and co-workers (1998) found that 57/77 (74%) carcinomas analyzed were positive for HLA class I expression and 10/77 (13%) also scored positive for HLA-DR expression. Goepel and co-workers (1991) analyzed 20 tumor samples. Total HLA class I expression was lost in 3 cases (15%), reduced in 2 (10%), and normal in the remaining 75%. Of 8 A2-positive samples, A2 expression was noted in 75% of the cases (1 normal and 5 reduced cases). Lucin and co-workers (1994) studied 75 ductal invasive carcinomas. In 45% of the cases, a heterogeneous staining pattern was noted, and 29% of the tumors were classified as completely negative for HLA expression. Interestingly, while normal breast epithelium was consistently negative for class II expression, 33% of the tumors were class II positive. Kaklamanis and co-workers (1995) studied 63 patients with lymph node metastasis at surgery of the primary tumor. Total losses were seen in 33% of primary tumors and 44% of secondary tumors. A2 selective loss was seen in 20% of the cases in both primary and metastatic tumors. Redondo and co-workers (1997a, 1997b) studied 16 benign lesions and 84 breast carcinomas by immunohistochemistry. All benign lesions were class I positive and 18/21 (86%) well-differentiated tumors were also positive. Similarly, moderately differentiated tumors were positive in 81% of the cases (31/38). By contrast, only 6/25 (24%) of poorly differentiated tumors were HLA positive. A separate study from Concha and co-workers (1991) evaluated 94 carcinomas for class I expression. All tumors derived from stage I patients were HLA^+, with most scoring as 2+ or better in the authors' scoring system. Eight (9%) samples were scored as HLA-negative tumors. Disease stages of these eight were II (3), III (3), and IV (2). An additional 23 (25%) were scored as +/–. K. Lyerly's group (personal communication) looked at class I expression on circulating tumor cells in seven breast cancer patients whose tumors were defined as $cytokeratin^+$, $Her2/neu^+$ and all had good levels of class I MHC (100%).

In conclusion, with the exception of poorly differentiated carcinomas, breast cancer appears to be mostly class I positive, with varying estimates in the 56–100% range (average 79%). In addition, A2-specific loss can occur in up to 20–25% of the cases.

Colon cancer

Goepel and co-workers (1991) described an analysis of 20 tumor samples. Class I HLA expression was totally lost in 2 cases (10%), reduced in 10 (50%), and normal in the remaining 40%. Of eight samples from A2-positive patients, A2 expression was noted in 25% of the cases. Klein and co-workers (1996) studied 90 biopsies. All well-differentiated tumors were highly positive for class I expression, and 80% of the moderately differentiated tumors were weakly positive. By contrast, none of the poorly differentiated tumors were found to express class I molecules. A different study (Rees et al. 1988) reported that approximately one-third of human colorectal carcinomas fail to express the HLA-A, -B, -C monomorphic determinant reactive with the W6/32 monoclonal antibody (mAb), and 44% express class II HLA antigens as shown by reactivity with NFK-1 mAb. In addition, loss of the A2 haplotype was shown in 4 of 15 tumor tissue samples (from the authors' abstract). However, Möller and Hämmerling (1992) reported that 10–15% of colon carcinomas have been repeatedly confirmed to lack W6/32 reactivity. In a further study, 70 colorectal tumors were stained (Browning et al. 1996). Complete absence was noted in 13% of the cases, and focal loss of expression in 4% of the tumors. In addition, selective loss of one HLA molecule was noted in 11% of the cases. These same authors noted that other authors have reported similar frequencies. Furthermore, IFN-γ treatment was noted to result in upregulation of class I molecules in the majority of the cases. In conclusion, with the exception of poorly differentiated tumors, colon cancers appear to be mostly class I positive, with varying estimates in the 67–100% range (average 85%). The references cited above suggest that no consensus exists regarding the frequency of HLA-A-specific loss (11–75% range).

Lung cancer

A classic study by Redondo and co-workers (1991) analyzed a total of 115 broncogenic carcinomas. Total loss of class I was detected in 29 (25%). In addition, selective loss of the *A* and *B* loci were seen in 3 and 5% of the cases, respectively. Class II staining was noted in 13/66 cases analyzed (18%). Subsequently, a series of 93 resection specimens of non-small-cell lung carcinomas was analyzed by immunohistochemical methods using various antibodies (Kaklamanis et al. 1995; Korkolopoulou et al. 1996). Eighty-six patients were included in the survival analysis. Total loss of class I molecules was observed in 38% of the cases. Selective loss of the *A* locus was seen in 8.3% and of the *A2* allele in 27% of the cases. No correlation was found between the expressional status of any of the above molecules, including the selective *A2* allelic loss and histological type, degree of differentiation, tumoral stage, nodal stage, and survival (from the authors' abstract). Redondo and co-workers (1997a, 1997b) analyzed a panel of broncogenic carcinomas. Of 52 tumors analyzed, 32 were squamous carcinomas, 16 were adenocarcinomas, 1 was a large-cell carcinoma, and 3 were small-cell carcinomas. Frozen sections were stained with mAbs and 66% of the tumor cells were found to be positive for class I expression. Hiraki and co-workers (1999) reported haplotype loss in 3/7 (43%) lung cancer cell lines analyzed. In conclusion, lung cancers appear to be mostly class I positive, with varying estimates in the 62–75% range (average 68%). The one reference cited above suggests that in addition, A-specific loss can occur in up to 27% of the cases.

Prostate cancer

Sanda and co-workers (1995) reported that three of five (60%) prostate tumor-derived cell lines expressed class I, and also noted that IFN-γ could upregulate class I expression by most (positive and negative) cell lines in vitro. Klein and co-workers (1996) reported that 88% of well-differentiated tumors but only 16% of poorly differentiated ones stained with anti-β_2-microglobulin antibodies. Blades and co-workers (1995) analyzed HLA class I and II expression in benign prostate hyperplasia, primary prostate cancers, and metastases by standard immunohistochemistry methods. Loss of class I staining occurred in 6/17 primary cancers (35%) and in 14/33 (42%) metastatic tumors. Of those tumors retaining class I expression, allele-specific loss occurred in 21/27 (78%) of the cases, with A2 being lost in 10/14 cases (71%). The authors comment that these frequencies are higher than those observed in other tumor types, suggesting immunoselective pressure being applied to the tumor. Bander and co-workers (1997) reported loss of class I in 50% of the prostate cancer specimens studied (a total of 18). In the remaining 50%, normal and reduced expression were noted at approximately similar frequencies. Finally, a study by Lu and co-workers (1999) analyzed a total of 34 prostatic adenocarcinomas. Total loss was detected in 21% of the cases. Of the remaining specimens, reduced or heterogenous expression was noted in 68%, while normal expression was noted in 9% of the samples. In conclusion, poorly differentiated prostate tumors are frequently class I negative. Well-differentiated (primary and metastatic) prostate tumors appear to be mostly class I positive, with varying estimates in the 88–50% range (average 66%). The one reference cited above suggests that in certain cases, A-specific loss can occur in up to 70% of the cases.

Cervical carcinoma

Allele-specific HLA class I and II expression on normal (n=10), premalignant (n=25), and malignant cervical tissue (n=30) was investigated by Hilders and co-workers (1994). No alterations in monomorphic or locus/allele-specific HLA class I or II expression were observed in normal and premalignant epithelial tissue. In cervical carcinomas, however, reduced expression of HLA class I antigens was present in 70% of the cases, comprising a monomorphic class I loss in 20%, and an allele-specific loss in 50% of HLA-A2-positive patients. De novo expression of class II antigens was observed in 80% of the cervical carcinomas, with the sublocus products being expressed in the order HLA-DR>HLA-DQ>HLA-DP. The authors' results show that alteration in HLA expression is a process confined to malignant cells, which may allow tumors to evade immune surveillance (from the authors' abstract).

van Driel and co-workers (1996) analyzed cervical carcinomas from 49 patients. In total, 97% of the cells were positive for class I expression (63%: normal levels of staining; 34%: heterogeneous staining). In addition, 67% of the cells were associated with positive staining for HLA class II. Normal staining was detected in 30% of the cases, while HLA-A2 was found to be selectively downregulated in 44% of the samples derived from A2 individuals. Thus, the vast majority (74%) of the samples retained expression of A2. Total A2 loss was observed only in 26% of the specimens derived from A2-positive individuals.

Koopman and co-workers (1998) analyzed in detail the possible alterations in HLA expression of five different cervical carcinoma-derived cell lines. Loss of expression was seen in three cases, in one of which, expression could be restored by IFN-γ treatment. Weak but positive A2 expression was noted for the two lines derived from the two A2-positive individuals, and expression levels could be further enhanced by IFN-γ treatment. In a subsequent study, the same

authors (Koopman et al. 2000) used flow cytometry and molecular analyses to concurrently assess allele-specific HLA phenotypes and genotypes in subpopulations from 30 freshly isolated cervical tumors. Tumor-associated HLA class I alterations were present in 90% of the lesions tested, comprising four altered pheno/genotype categories: (1) HLA-A or -B allelic loss (17%); (2) HLA haplotype loss, associated with loss of heterozygosity at 6p (50%); (3) Total HLA class I antigen loss and ROH at 6p (10%), and (4) *B* locus or HLA-A/B downregulation associated with ROH and/or allelic imbalance at 6p (10%). In conclusion, cervical carcinomas appear to be mostly class I positive, with varying estimates in the 40–97% range (average 77%). The references cited above suggest that, in addition, *A2* locus-specific loss can occur in 0–50% (average 32%) of the cases.

Other tumor types

Torres and co-workers (1996) studied 25 pancreas cancers. They noted that loss of expression of individual alleles, which is the most frequently detected alteration, is most consistent with an immunoselection process. A similar point was raised by Kaklamanis and Hill (1992). The frequencies of alterations they reported are difficult to interpret, because in certain cases (see their Table I, for example) loss of W6/32 reactivity is associated with normal HLA-A and HLA-B reactivity. Some of reported results are likely to have been affected by technical difficulties.

Nevertheless, the authors note that lack of W6/32 reactivity is, in general, correlated with less-differentiated tumors. A different study was performed by Scupoli and co-workers (1996), which analyzed HLA expression in eight different carcinomas. Loss of HLA expression was detected in two of the eight (25%) cases. A study by Cromme and co-workers (1994) reported loss of class I in 31/76 cases (41%). Klein and co-workers (1996) noted lack of staining in 10–70% of testicular cancer tumors, and noted that reduced expression correlated with both tumor aggressiveness and disease stage. In the case of renal cell carcinoma, in localized disease, lack of class I expression may correlate with poor prognosis. Gastl and co-workers (1996) reported 100% of primary and 82% of metastatic renal carcinomas as positive for class I expression.

According to a study from Esteban and co-workers (1996), 21% of larynx tumors analyzed were associated with total class I loss, 8% had loss of HLA-A only, while 1% had loss of HLA-B only. Interestingly, no difference in the composition of the tumor infiltrate (based on either NK cells, macrophages, CD3, CD4, and CD8 cells) was noted between tumors classified as HLA class I positive or negative on the basis of immunohistochemistry. These results raised the possibility that enough class I molecules were expressed within tumors scoring as negative in the histological readout to attract T cells to the tumor and cause the infiltration of immune cells. Finally, decreased expression was reported in 51% of melanomas, with total loss limited to 16.1% (Ferrone and Marincola 1995). A different study (Restifo et al. 1996) reported loss of class I expression in 5/13 (31%) of melanoma patients receiving immunotherapy.

These figures are not dissimilar to those reported in the preceding sections for breast, colon, lung, cervical, and prostate cancers.

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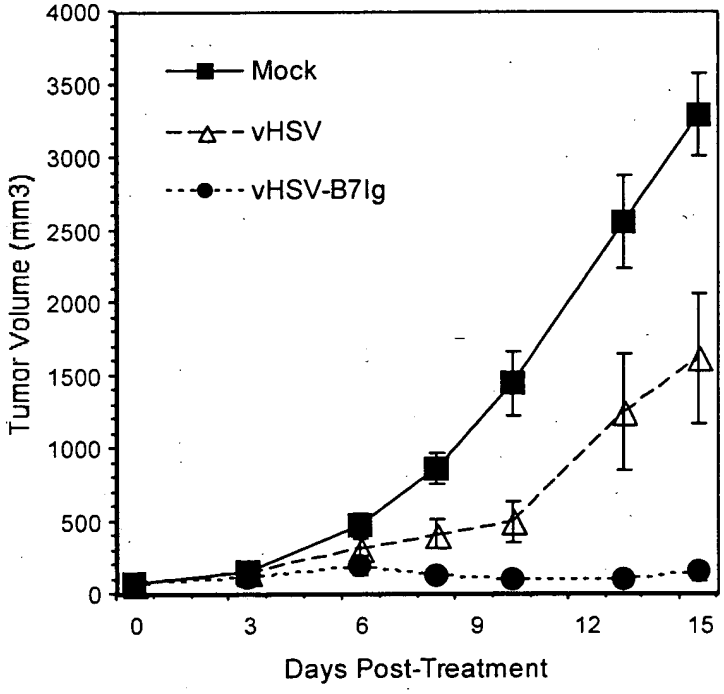

Figure. Anti-tumor activity of recombinant oncolytic HSV vector expressing B7-Ig. Established subcutaneous Neuro2a tumors in A/J mice were treated with vHSV or vHSV-B7Ig (2 x 105 pfu) or mock on days 0 and 3 (n=7-9). Intraneoplastic injection of vHSV-B7Ig caused a significant inhibition of tumor growth compared with mock ($p<0.001$, day 15, unpaired t test) or vHSV, a control vector without soluble B7-1 expression ($p<0.05$, day 15).

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THE B7 FAMILY OF LIGANDS AND ITS RECEPTORS: New Pathways for Costimulation and Inhibition of Immune Responses

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Key Words ICOS, PD-1, PD-L, T cell activation, tolerance

■ **Abstract** T cell activation is dependent upon signals delivered through the antigen-specific T cell receptor and accessory receptors on the T cell. A primary costimulatory signal is delivered through the CD28 receptor after engagement of its ligands, B7-1 (CD80) or B7-2 (CD86). Engagement of CTLA-4 (CD152) by the same B7-1 or B7-2 ligands results in attenuation of T cells responses. Recently, molecular homologs of CD28 and CTLA-4 receptors and their B7-like ligands have been identified. ICOS is a CD28-like costimulatory receptor with a unique B7-like ligand. PD-1 is an inhibitory receptor, with two B7-like ligands. Additional members of B7 and CD28 gene families have been proposed. Integration of signals through this family of costimulatory and inhibitory receptors and their ligands is critical for activation of immune responses and tolerance. Understanding these pathways will allow development of new strategies for therapeutic intervention in immune-mediated diseases.

THE B7/CD28/CTLA-4 PATHWAY: THE PARADIGM FOR THE FAMILY

The CD28/CTLA-4/B7-1/B7-2 family provides a paradigm with which to define new related immune pathways. From this pathway, we find that multiple B7 ligands bind to both activating (CD28) and inhibitory (CTLA-4) receptors (1–3). These receptors do not function independently, but they modify responses delivered by engagement of the antigen-specific TCR on T cells. To date, members of the receptor family are type I transmembrane proteins with a single IgV extracellular domain, and the ligands are type I transmembrane proteins with both IgV and IgC extracellular domains. Interactions between the receptor-ligand pairs are mediated predominantly by residues in the IgV domains. Expression of both receptors and ligands is tightly regulated, allowing discrimination between signals that result in activation or inhibition of an immune response.

The CD28 receptor is constitutively expressed on T cells. Engagement of CD28 on naive T cells by either B7-1 or B7-2 ligands on antigen-presenting cells

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provides a potent costimulatory signal to T cells activated through their T cell receptor (1–3). This results in induction of IL-2 transcription, expression of CD25, and entry into the cell cycle. CD28 engagement also confers critical survival signals to T cells through the Bcl-X_L pathway (4). CD28 costimulation is necessary for the initiation of most T cell responses, and blockade of CD28 signaling results in ineffective T cell activation. This has therapeutic implications, in that blockade of CD28 costimulation can be profoundly immunosuppressive, preventing induction of pathogenic T cell responses in autoimmune disease models and allowing for prolonged acceptance of allografts in models of organ transplantation (1, 2).

CTLA-4 (CD152) shares about 30% identity with CD28 at the amino acid level (Table 1). CTLA-4 expression is not detected on naïve T cells but is transcriptionally induced after T cell activation (3). Cell surface expression of CTLA-4 is very tightly regulated, with most of the CTLA-4 protein residing within cytoplasmic vesicles (5). The critical role of CTLA-4 as a negative regulator of T cell activation is dramatically illustrated in CTLA-4-deficient mice, which die within 3 to 4 weeks of birth from massive lymphocytic infiltration and tissue destruction in critical organs (6–8).

Both CD28 and CTLA-4 share binding to B7-1 (CD80) and B7-2 (CD86) ligands. B7-1 and B7-2 are capable of forming homodimers, allowing for interactions with homodimers of either CTLA-4 or CD28. The interaction of CD28 with its ligands is weaker than the interaction with CTLA-4. Human B7-1 binds to human CTLA-4 and CD28 with Kd values of 0.42 and 4 μM, respectively for the monomeric interactions (9). The B7-2:CTLA-4 interaction is of an affinity similar to B7-1:CD28, and the CD28:B7-2 interaction is of even lower affinity (10). Mice

TABLE 1 B7 family receptors: Amino acid identities (%) were calculated using the Wisconsin Package (GCG) Version 10 Gap program, and the Blosum 62 scoring matrix. For sequence comparisons indicated with an asterisk, the Structgappep scoring matrix was used, as the sequences were too distantly related to align with Blosum 62. Comparisons between all mouse (m) and human (h) proteins are shown

	Protein identities (%) among B7 family receptors						
Protein	**mCD28**	**hCTLA-4**	**mCTLA-4**	**hICOS**	**mICOS**	**hPD-1**	**mPD-1**
hCD28	69	33	34	29	29	15*	18*
mCD28		30	32	24	25	12*	20*
hCTLA-4			74	18	18	20	18*
mCTLA-4				21	18	22	18*
hICOS					69	13*	16*
mICOS						12*	14*
hPD-1							60

deficient in B7-1 and B7-2 have significant abnormalities in both humoral and cellular immune responses, again illustrating the fundamental role of this pathway (11).

The recent crystal structures of CTLA-4-B7 complexes are characterized by homodimers of CTLA-4 that contain B7-binding sites located distally to the CTLA-4 dimer interface (12, 13). The crystal structures suggest that the CTLA-4 homodimer can bind to noncovalent homodimers of B7-1 or B7-2 to form a lattice of CTLA-4-B7 interactions (12, 13). Formation of such a lattice could function to form a stable signaling complex at the T cell–APC interface. CD28 also forms homodimers, with a conserved cysteine located just proximal to the transmembrane domain linking the monomers in CD28 and CTLA-4. By homology, CD28 may also form lattice structures with B7-1 and B7-2, which could serve to potentiate the costimulatory signals delivered through CD28.

With the determination of the human genome sequence, and with significant accumulation of mouse genomic sequences, algorithms for gene homology can be used to identify genes encoding proteins with structural homology to B7 and CD28/CTLA-4. The Ig superfamily represents a large number of proteins, including BCR and TCR, and thus it does not provide sufficient criteria to identify a protein as a new costimulatory or inhibitory receptor or ligand. Instead, proteins with the highest homology to known gene family members are identified and tested for functional relationships. In addition, gene mapping can suggest evolutionary relationships. Recently, several proteins have been identified as new members of the B7 and CD28/CTLA-4 families (Figure 1). The B7 family ligands have been selected based on identities of about 20% to 30% in the extracellular domains, and they are characterized by an amino terminal signal peptide, one Ig-V and one Ig-C domain, a transmembrane domain, and a cytoplasmic tail. Inclusion in the B7 family of ligands is based upon degree of homology, as well as on evidence of costimulatory or inhibitory function in immune assays. New transmembrane proteins related to the CD28 receptor family have been identified based upon homology, IgV extracellular domain structure, functional activity, and binding to a B7-like ligand.

THE ICOS PATHWAY: IDENTIFICATION OF ICOS AND ITS LIGAND

ICOS (AILIM) is a costimulatory receptor homologous to CD28 and CTLA-4 (14, 15) (Table 1). Human ICOS is a 55–60 kDa, disulfide-linked, glycosylated homodimer when isolated from activated human T cells. The protein has two putative N-glycosylation sites, and the unglycosylated monomer has a molecular weight of about 20 kDa (16). Mouse ICOS is a 47–57 kDa, disulfide-linked, N-glycosylated homodimer (17). Rat ICOS is highly homologous (85% identity) to the mouse protein (18). In rat, a differentially spliced form of ICOS containing a longer cytoplasmic tail has been identified, which suggests that additional forms may exist in human and mouse (18).

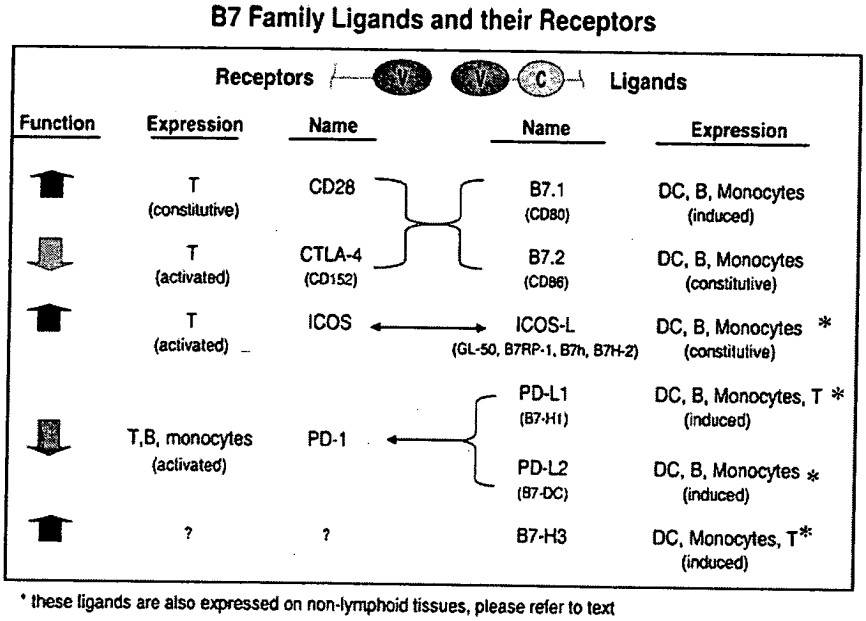

Figure 1 Summary of B7 family ligands and their receptors: The names of receptors and ligands are indicated, as well as a brief summary of predominant expression patterns for each. The conserved structure of a single IgV extracellular domain for receptors and IgV and IgC extracellular domains for ligands is depicted at the top. Function arrows summarize whether the pathway is thought predominantly to costimulate or inhibit the response of the receptor-bearing cell.

ICOS lacks the canonical MYPPPY motif, which is present in the extracellular domains of CD28 and CTLA-4, but contains a related FDPPPF sequence in the analogous position in the protein. The crystal structures of the CTLA-4/B7-1 and CTLA-4/B7-2 complexes implicate the MYPPPY site as the major contact site in CTLA-4 with B7-1 and B7-2 (12, 13). Although the structure of CD28 has not yet been solved, amino acid homologies, mutation data, and modeling support the concept that this motif will also be a major B7-binding site for CD28 (19). The related FDPPPF site in ICOS is not sufficiently conserved to allow for detectable binding of ICOS to B7-1 or B7-2 (16, 20, 21). However, structural homology raises the possibility that this motif might be important in the binding of ICOS to its ligand, which is a member of the B7 family (see below).

The ICOS gene is closely linked to the genes for CD28 and CTLA-4 on human chromosome 2q33 and mouse chromosome 1 (17, 22). In humans, the three genes form a tightly linked cluster, with a gene order of CD28-CTLA-4-ICOS within a 300-kb region (22a), suggesting that these genes originated by gene duplication. This tight clustering suggests that expression of these genes may be coordinately

regulated, with alterations in chromatin structure affecting the entire locus, as observed for tightly linked cytokine genes (23). Moreover, disease susceptibility loci mapped to this region must include ICOS in addition to CD28 and CTLA-4 as candidate genes.

ICOS is an inducible costimulatory receptor expressed on activated, but not resting, T cells (14); ICOS is identical to the H4 T cell activation antigen (24). Expression of ICOS on T cells is dependent upon both TCR and CD28 signals, in that activation of T cells in the absence of CD28 engagement results in diminished levels of ICOS on T cells (25). This suggests that signals delivered by ICOS will typically occur distally to those delivered through CD28. However, ICOS expression is not absolutely dependent upon CD28 signals because activated human $CD8^+$ T cells that do not express CD28 can express ICOS (14), and some T cell responses in CD28-deficient mice can be modulated with ICOS.Fc (26). Interestingly, although blockade of the CD28-B7 interaction opposes induction of ICOS expression, blockade of the CD40-CD40L pathway has no effect on ICOS induction on activated human T cells (16).

The costimulatory functions of both CD28 and ICOS raise the possibility that they may share overlapping signaling pathways. The cytoplasmic tail of CD28 contains a YMNM motif, which is a PI-3 kinase binding site, and which can bind Grb2 and a Grb2-related protein GADS/GRID (27). Mutations in this site result in a failure to effectively recruit PI3K and a failure to induce Bcl-X_L expression in response to CD28 ligation (27, 28). In addition, a consensus SH3-kinase binding site, PYAP, distal to the YMNM site is critical for costimulation of proliferation and IL-2 production (28, 29). The cytoplasmic tail of ICOS contains a YMFM motif, which binds the p85 subunit of PI3K, although binding of Grb-2 to ICOS was not detected (22). The cytoplasmic tail of ICOS lacks the PXXP site implicated in IL-2 production by CD28 engagement, which may account, in part, for the distinct functions of CD28 and ICOS.

ICOS is expressed on T cells in lymphoid organs, such as spleen, lymph node, and Peyer's patches in human and mouse (14, 16, 17, 21). $ICOS^+$ T cells are found in germinal centers and surrounding T cell zones, and ICOS expression in these areas is enhanced after immune priming (21). $CXCR5^+$ T cells, a subset of $CD4^+$ T cells that are found in B cell follicles and germinal centers, are highly enriched in ICOS expression, as compared with T cells from peripheral blood (30, 31). This activated T cell subset is likely to be involved in enhancement of antibody responses.

ICOS is expressed in the medulla and the cortico-medullary junction of the thymus (17). However, mice deficient for ICOS have a normal thymus and normal numbers of peripheral $CD4^+$ and $CD8^+$ T cells, suggesting that ICOS does not play a critical role in T cell development (32–34). In human, ICOS expression was detected in fetal and newborn thymuses, with expression primarily in the medulla (16). These data suggest that ICOS could contribute to thymic development, but analysis of ICOS-deficient mice indicates that ICOS is not obligatory. Similarly, mice deficient in CD28 have normal thymuses and normal numbers of peripheral

TABLE 2 B7 family ligands: Amino acid identities (%) were calculated using the Wisconsin Package (GCG) Version 10 Gap program, and the Blosum 62 scoring matrix. Comparisons between all mouse (m) and human (h) proteins are shown. At the time of this review, the sequence for mouse B7-H3 was not available

	Protein identities (%) among B7 family ligands									
Protein	**mB7.1**	**hB7.2**	**mB7.2**	**hICOS-L**	**mICOS-L**	**hB7H3**	**hPD-L1**	**mPD-L1**	**hPD-L2**	**mPD-L2**
hB7.1	45	26	24	26	26	27	25	25	23	24
mB7.1		30	27	24	27	26	26	25	24	25
hB7.2			51	23	27	26	21	26	22	20
mB7.2				25	25	28	24	24	20	21
hICOS-L					48	33	25	27	27	28
mICOS-L						31	25	25	22	29
hB7H3							31	28	27	26
hPD-L1								70	41	39
mPD-L1									43	38
hPD-L2										70

T and B cells, indicating that these costimulatory pathways are not obligatory for normal T cell development (35).

The ligand for ICOS (B7h, GL50, B7RP-1, LICOS, B7-H2, KIAA0653), which is called ICOS-L for clarity and nonpartisanship in this review, was identified as a B7-like molecule capable of binding to ICOS and delivering a costimulatory signal to T cells (17, 20, 21, 36–38). Comparisons indicate that mouse and human ICOS-L share about 48% amino acid identity, and about 25% identity with other members of the B7 family (Table 2). Human ICOS-L maps to chromosome 21q22.3 and has been annotated on the chromosome 21 DNA sequence at position 31156109 (39, 40). The location of the human gene suggests that mouse ICOS-L should map in the syntenic region of mouse chromosome 10. Thus, in contrast to the CD28/CTLA-4/ICOS gene cluster, ICOS-L is in a distinct location from the B7-1 and B7-2 genes, which are on human chromosome 3q13.3-21 and mouse chromosome 16, respectively (41). Interestingly, the transcripts identified by the different groups as ICOS-L are not identical but result from differential splicing patterns (42). Two predicted forms of the human protein have been identified from immune sources, encoding proteins with differences in their cytoplasmic tails. It will be interesting to determine whether there are functional implications for this differential splicing. Differential splice variants have also been observed for the B7-1 and B7-2 genes (43–45).

Measurement of the binding affinity of ICOS to ICOS-L indicates that the affinity of this interaction is very comparable to that of CD28 and B7-1 (21, 38). Davis and colleagues (38) measured a Kd of 4 μM for the interaction of monomers of ICOS-L with immobilized ICOS.Fc at 37°C, similar to their estimates of the affinities for B7-1 and CD28 (9, 38). Although they detected weak binding of tetramers of ICOS-L to CD28 and CTLA-4 at 25°C, there was no binding of monomers at 37°C; other groups have failed to detect this association using dimeric

reagents. This suggests that ICOS-L is not a physiological ligand for CD28 or CTLA-4.

ICOS-L is expressed constituitively on unstimulated B cells, splenic and peritoneal macrophages, and peripheral blood–derived dendritic cells by binding of ICOS.Fc to murine cells (20, 21, 36). It has also been detected on a small subset of $CD3^+$ T cells, like other B7-family members (20). In humans, ICOS.Fc bound to peripheral B cells from some donors, and to monocytes and monocyte-derived dendritic cells (46). Interestingly, INFγ, but not LPS or TNFα or anti-CD40L, augmented the expression of ICOS-L on $CD14^+$ monocytes. ICOS-L expression has been detected by analysis of mRNA in many nonlymphoid tissues, such as kidney, liver, heart, and brain (20, 36). TNFα further induces expression of ICOS-L on B cells and monocytes, and most interestingly, it induces the expression of ICOS-L on fibroblasts by mRNA analysis. LPS treatment of mice, which induces TNF-α production, induced ICOS-L mRNA expression in testes, kidney, and peritoneum (36). ICOS-L expression was downmodulated in spleen cells from mice treated with LPS. These data suggest that ICOS-L may be induced by inflammatory signals in peripheral sites, although this must be confirmed at the protein level. Examination of ICOS-L protein in rejecting heart transplants showed that ICOS-L protein was expressed by interstitial dendritic cells in normal myocardium, and additionally on large inflammatory macrophages in rejecting hearts (47).

MODULATION OF IMMUNE RESPONSES BY THE ICOS PATHWAY

Engagement of ICOS on T cells that have been stimulated through the TCR results in augmented proliferative responses and cytokine production (14). In comparisons of costimulation mediated through CD28 and ICOS, production of IL-2 is most effectively induced by CD28 (21, 48, 49), although modest enhancement of IL-2 production by ICOS engagement has been reported (37, 50). Costimulation of human $CD4^+$ T cells by ICOS does not produce sustained proliferative responses due to limiting IL-2 production (50). Costimulation through ICOS is particularly effective in enhancing IL-10 production; in direct comparisons, ICOS is more potent than CD28 in inducing the production of IL-10 (14, 16, 25, 37). Both pathways augment the production of other effector cytokines such as IFNγ, IL-4, IL-5, and TNFα (25, 49, 50). Thus, CD28 costimulation appears to have a nonredundant role in the initial costimulation of IL-2 and is critical for initiation of immune responses. In contrast, the subsequent expression of ICOS and engagement by ICOS-L is more important for augmentation of IL-10 and enhancement of effector functions.

ICOS engagement can augment induction of both Th1 and Th2 cytokines, but under some circumstances it may more effectively costimulate Th2 responses. ICOS is expressed similarly on both Th1 and Th2 lines after primary stimulation but remains high only on Th2 lines after repeated activation steps (22, 25). This

suggests that blockade of the ICOS pathway could affect both primary Th1 and Th2 responses, but that highly polarized Th2 responses might be more affected by ICOS blockade. Kopf et al. (26) show that both the Th1 cytokine IFNγ and Th2 cytokines IL-4 and IL-5 are reduced by administration of ICOS.Fc at the time of infection with *N. brasiliensis*, indicating that the ICOS pathway can enhance production of both Th1 and Th2 cytokines in vivo. In addition, Coyle et al. (22) show that production of both Th1 and Th2 cytokines can be suppressed by addition of ICOS.Fc at the time of reactivation in vitro. However, for highly polarized lines, only the production of Th2 cytokines is reduced by ICOS blockade (22). In addition, adoptive transfer of polarized Th2 lines in a lung inflammatory model results in a dependence upon ICOS engagement in vivo for optimal production of Th2 cytokines and resulting eosinophilia. The highly polarized Th1 line, which induces a neutrophilic infiltrate, is not ICOS dependent (22). Thus, it appears that Th2 cytokine production will generally be more dependent upon ICOS costimulation, whereas the dependence upon ICOS for Th1 cytokines will be determined by the precise conditions under which that Th1 response is elicited. Recent studies in EAE suggest that ICOS costimulation may play a larger role in the effector phase of a Th1 response, in that disease is ameliorated by blockade of ICOS only during the effector phase (51). CD28 costimulation augments production of both Th1 and Th2 cytokines as well, but Th2 responses are also more dependent upon CD28 costimulation (1). Thus, it may be that Th2 responses simply require a higher threshold of costimulatory signals, and that both CD28 and ICOS contribute to the induction of Th2 responses. Interestingly, CTLA-4 engagement can oppose T cell activation with costimulatory signals delivered by either CD28 or ICOS (50), and thus the outcome of the immune response will also depend upon whether CTLA-4 is engaged concomitantly with ICOS by APC that express both B7 ligands and ICOS-L.

ICOS engagement can also influence $CD8^+$ T cell responses. Expression of ICOS-L in an immunogenic, MHC class I^+ tumor resulted in enhanced tumor rejection in mice (52). In these studies, ICOS-L costimulation of $CD8^+$ T cells was found to enhance IL-2 and IFN-γ production preferentially in recall responses compared with naive responses. No enhancement of $CD8^+$ lytic effector function was observed (52), which is consistent with studies showing that inhibition of the ICOS pathway had no effect on CTL responses after LCMV or VSV infection in mice (26). Thus, generation of lytic effector functions in $CD8^+$ T cells does not appear to be ICOS-dependent.

The ICOS pathway appears to play a large role in antibody responses and germinal center formation. ICOS is expressed by germinal center T cells, and its ligand is expressed by splenic B cells (21). Transgenic mice expressing a secreted form of ICOS-L.Fc protein are characterized by lymphoid hyperplasia in the spleen, lymph nodes, and Peyer's patches, and have high serum levels of IgG (21). Evaluation of ICOS-deficient mice by three independent groups supports a critical role for ICOS in humoral immunity (32–34). ICOS-deficient mice have a consistent decrease in serum IgG1 levels (32, 34), and immunization of mice with TNP-KLH in the absence of adjuvant or with alum or IFA reveals a deficit in IgG1 and IgG2a antibody

production (32). This deficit could be overcome by the use of CFA as an adjuvant (32). However, in another study, immunization with KLH in CFA also resulted in decreased isotype switching (34), suggesting that the precise immunization conditions and antigen used may affect the outcome. Immunization of mice with NP-OVA in alum (34) or with aerosolized antigen in the lung (33) also revealed a deficit in IgE production in ICOS-deficient mice. Thus, under several conditions of immunization, deficits in isotype switching have been observed. This deficit is not rescued by secondary immunization and appears to be due to a lack of T cell help, as isotype switching to T cell–independent antigens is intact (34). Activation of CD40 was able to rescue the defect in isotype switching in ICOS-deficient mice (32). Engagement of ICOS can enhance anti-CD3-mediated induction of CD40L on T cells, indicating that both CD28 and ICOS can contribute to activation of the CD40L-CD40 pathway. ICOS-L is highly expressed in the B cell–rich areas of the spleen (21), consistent with the proposal that activation of the CD40 pathway may be the critical event mediated by the ICOS pathway in the development of a humoral response (32). However, expression of CD40L is clearly not absolutely dependent upon ICOS expression, as ConA-activated T cells from ICOS-deficient mice can express normal levels of CD40L (53).

Consistent defects in germinal center formation are also observed in ICOS-deficient mice (32–34). Mice form fewer and smaller germinal centers in response to both primary and secondary immunization (32–34). Mice deficient in CD28 (35) or in both B7-1 and B7-2 (11) are also defective in isotype switching and germinal center formation. Similarly, mice deficient in CD40L have severe deficits in isotype switching and germinal center formation (54, 55). It will be interesting to determine precisely how these three pathways intersect in directing the humoral immune response.

THERAPEUTIC IMPLICATIONS: THE ICOS PATHWAY

The apparent increased dependence of Th2 responses on the ICOS pathway and the clear role for ICOS in isotype switching raise the possibility that targeting this pathway may be useful in generation of therapeutics for diseases with antibody-mediated and Th2-mediated pathologies. Interestingly, antagonism of ICOS appears to be more effective late in an immune response. Blockade of ICOS at the time of antigen priming for lung inflammation had little effect upon subsequent airway challenge in normal mice (49). In addition, ICOS-deficient mice are still susceptible to induction of inflammatory lung disease induced by airway challenge with OVA in primed mice (33). The absence of ICOS in this model results in lower production of IL-4 and IL-13, but no change in the lung histology (33). In contrast, antagonism of ICOS 21 days after priming significantly reduced lung inflammation after airway antigen challenge (49). Similarly, ICOS-blockade decreased lung inflammation and airway hyperreactivity after adoptive transfer of highly polarized Th2 cells to naive mice (22). Blockade of the B7/CD28 pathway

also prevents airway hyperreactivity after adoptive transfer of either Th1 or Th2 cells lines and, further, does not show the Th2-bias of the ICOS blockade. Antagonism of CD28 is more effective at the time of antigen priming and less effective at later times in these models of lung inflammation (49). This suggests that the major contributions of CD28 and ICOS in costimulation of Th2 responses occur at different times during generation of this response.

Induction of EAE, a Th1-mediated autoimmune disease, is not dependent upon ICOS, in that antagonism of ICOS at the time of antigen priming does not prevent disease (51). Surprisingly, ICOS-deficient mice are more susceptible to EAE (33), and blockade of ICOS at the time of priming for EAE results in more severe disease (51). The enhanced disease could be due to the absence of a protective Th2 response, as a deficiency in IL-13 production was noted (33). Other explanations are possible at this time, including defects in IL-10 production or defects in regulatory cells, both of which would be expected to result in enhanced disease (56, 57). $CD4^+$ $CD25^+$ regulatory cells are dependent upon CD28 for maintenance in the periphery (58) but have not yet been examined in ICOS-deficient mice. ICOS costimulation in EAE does appear to be critical at the time that encephalitogenic T cells begin to migrate into the CNS (51). Antagonism of ICOS at this time reduced disease severity. Mice deficient in CD28 or in both B7-1 and B7-2 are resistant to EAE (59). Interestingly, adoptive transfer of primed encephalitogenic T cells into B7-double deficient mice also results in reduced disease, implicating CD28 engagement in both priming and effector stages of the disease (59).

The ICOS pathway plays a role in graft rejection, in that blockade of the ICOS pathway results in prolongation of heart allograft survival in mouse models (47). Combination of anti-ICOS antibody and anti-CD40L antibody in this heart transplant model also reduced vasculopathy in the cardiac grafts compared with anti-CD40L alone, suggesting that the ICOS pathway is contributing to chronic allograft rejection (47). Similarly, combinations of agents that block the B7/CD28 pathway and anti-CD40L promote long-term cardiac allograft survival in mice and prevent development of vascular lesions associated with chronic rejection (60). These data suggest that both ICOS and CD28 are contributing to inflammatory stimuli underlying the chronic rejection pathology. IL-4 production has been linked to development of transplant arteriosclerosis in mice deficient for CD40 (61). It is possible that both CD28 and ICOS contribute to chronic rejection pathology by costimulation of Th2 responses that could accompany the Th1 response mediating graft rejection. In addition, these pathways may function in promoting alloantibody responses, which could contribute to inflammatory responses in vessels.

THE PD-1/PD-L PATHWAY

PD-1 (program death-1) is a 50–55 kDa type I transmembrane receptor that was identified in a T cell line undergoing activation-induced cell death (62). PD-1 is a member of the Ig superfamily that contains a single Ig V–like domain in

its extracellular region (Table 1); it lacks the MYPPPY motif, a sequence critical for CTLA-4 and CD28 binding to B7.1 and B7.2 (63). The PD-1 cytoplasmic domain contains two tyrosines, with the most membrane-proximal tyrosine (VAYEEL in mouse PD-1) located within an ITIM (immuno-receptor tyrosine-based inhibitory motif) (62). The presence of an ITIM on PD-1 strongly suggested that this molecule could function to attenuate antigen receptor signaling by recruitment of cytoplasmic phosphatases (64).

Human and murine PD-1 proteins share 60% amino acid identity with conservation of four potential N-glycosylation sites, and residues that define the Ig-V domain (65, 66). The ITIM in the cytoplasmic region and the ITIM-like motif surrounding the carboxy-terminal tyrosine (TEYATI in human and mouse) are also conserved between human and murine orthologues. There is 62% amino acid identity in the PD-1 cytoplasmic region between human and murine proteins. This contrasts with the 100% conservation observed between human and murine CTLA-4 cytoplasmic regions. The genome location of human PD-1 has been mapped to chromosome 2q37.3 (65); CTLA-4, CD28, and ICOS mapped on the same chromosome at 2q33 (17, 22, 22a).

In normal murine tissue, PD-1 mRNA expression is confined to the thymus (67). Approximately 1% of thymocytes are PD-1 positive with expression restricted to a subset within the double negative (DN) population (67). In vivo administration of anti-CD3 mAb results in marked apoptosis of $CD4^+CD8^+$ (DP) cells in the thymus; however, such treatment leads to induction of PD-1 protein expression on the surviving $CD4^-CD8^-$ (DN) and single positive (SP) thymocytes. Significantly anti-CD3 mAb, but not dexamethasome, treatment induces PD-1 expression. These observations strongly suggest that T cell activation, and not induction of apoptotic death per se, results in expression of PD-1.

The potential role of PD-1 in thymic selection has been studied in detail (68). An increased percentage of PD-1^+ DN thymocytes has been reported in neutral and positively selecting backgrounds. Interestingly, PD-1 deficiency in positive selecting backgrounds resulted in an increase in DP cells and a decrease in SP thymocytes (68). These findings suggested a role for PD-1 in thymic positive selection, in which PD-1 engagement could increase the threshold of pre-TCR/CD3 complex signals required for transition from the DN to DP stage. In PD-1-deficient mice, a lower pre-TCR/CD3 complex threshold would allow for a higher number of cells to transition from DN to DP. In addition, PD-1 could also affect the efficiency of positive selection by modulating the threshold of TCR $\alpha\beta$ signals. In contrast, PD-1 deficiency has a negligible effect on negative selection of TCR transgenic T cells (69). These studies suggest that TCR thresholds of activation can be modulated upon engagement of PD-1. Moreover, these studies suggest that PD-1 plays no significant role in central tolerance.

Analysis of murine spleen and lymph node populations indicated that within each of these populations a small percentage of PD-1 positive cells could be detected (63). Under resting conditions, neither T nor B cells expressed PD-1. However, activation of T or B cells through the antigen receptor or with PMA and

ionomycin resulted in cell surface expression of the PD-1 receptor (63). PD-1$^+$ T and B cells are large in size and co-express CD25 and CD69, correlating expression of PD-1 with cell activation. PD-1 protein expression can be detected as early as 24 h after TCR activation. Both CD4$^+$ and CD8$^+$ T cell populations expressed cell surface PD-1 upon activation, and the pattern of PD-1 expression on human T cells is similar to that observed for murine T cells (B. Carreno, unpublished observations). PD-1 is also expressed in activated macrophages (70).

The ligands for PD-1 are the B7 family members PD-L1 (B7-H1) and PD-L2 (B7-DC) (71–74). Interaction of PD-1 with either PD-L1 or PD-L2 results in inhibition of T and B cell responses (70–72). An alternatively spliced PD-L2 variant lacking the Ig V–like domain has been described, but this variant does not bind to PD-1 (72). PD-L1 and PD-L2 share 40% amino acid identity and thus are more homologous to each other than to other ligands of the B7 family (Table 2). Human and murine orthologues of PD-L1 or PD-L2 share 70% amino acid identity. Interestingly, murine PD-L2 has only 5 amino acids in its cytoplasmic tail, whereas the human PD-L2 cytoplasmic region is 28 amino acids in length. Both human PD-L1 and PD-L2 genes map to chromosome 9p24.2, and these genes are separated by only 42 kb (72). This genomic proximity is reminiscent of that observed for B7-1 and B7-2, which are tightly linked on human chromosome 3q13.3-21 (41). Murine PD-L2 maps to a region located between 19C2 and 19C3 (74).

A variety of normal tissues have been examined for expression of PD-L1 and PD-L2 transcripts (71–73). The pattern of expression of these molecules is significantly broader than that reported for other B7 family ligands. The overall distribution of PD-L1 and PD-L2 transcripts is similar in human and murine tissues, with high levels of expression in placenta, low expression levels in spleen, lymph nodes, and thymus, and the absence of expression in brain. Transcripts for both PD-L1 and PD-L2 are detected in human heart; in murine hearts, transcripts for PD-L1 are abundantly expressed whereas PD-L2 transcripts are absent (71, 72). PD-L2 but not PD-L1 transcripts are detected in human pancreas, lung, and liver (72). Identification of the cellular populations that express PD-L1 and PD-L2 in these tissues awaits further investigation. Expression of PD-L1 and PD-L2 in both lymphoid and nonlymphoid tissues suggests that the PD-1/PD-L pathway may modulate immune responses in secondary lymphoid organs as well as in peripheral sites.

Expression of PD-L1 and PD-L2 on antigen presenting cell (APC) populations has also been examined in detail (71–73). Resting B cells, monocytes, and dendritic cells do not express either PD-L1 or PD-L2. Transcripts for these ligands can be detected upon activation of these populations by antigen receptor, LPS, or IFN-γ. In human B cell populations, LPS or BCR activation results in induction of PD-L1 and PD-L2 (71–73). In human monocytes, IFN-γ, but not TNF-α, treatment results in expression of both ligands; PD-L1 expression precedes that of PD-L2. Interestingly, IFN-γ treatment also upregulates B7-1 transcripts and ICOS-L protein expression on human monocyte populations (46, 71). On dendritic cells, LPS plus IFN-γ treatment induces PD-L1 and PD-L2 mRNA expression (71–73). B7-1 and B7-2 transcripts are also upregulated in these cells by LPS plus IFN-γ. Tseng

et al. (74) reported that PD-L2 (B7-DC) transcripts are exclusively expressed on spleen and bone marrow–derived murine dendritic cells. These authors postulate that some of the unique functions of dendritic cells could be attributed to PD-L2 expression. Further studies will be necessary to sort out these discrepancies in expression patterns.

Interestingly, mitogen or TCR activation of murine and human T cells results in cell surface expression of PD-L1 in addition to PD-1 (73) (L. Carter, B. Carreno, unpublished observations). Similarly, BCR-activated B cells express PD-L1 and PD-L2 in addition to PD-1 (63, 71, 72). Thus, B and T cell function can be modulated by engagement of cell-surface PD-1. Additionally, upon activation, both T and B cells can engage PD-1 on other cells through expression of PD-1 ligands. This suggests that at points of T:B contact, modulation of antigen receptor signals can occur bidirectionally through PD-1. The consequences of such an interaction are unknown but might serve to limit TCR and BCR receptor signaling after activation.

IFN-γ can also modulate PD-L1 expression in nonlymphoid cells. Endothelial cells constituitively express cell-surface PD-L1, and in vitro treatment with IFN-γ, but not LPS or TNF-α, results in its rapid upregulation (M. Eppihimer, J. Leonard, personal communication). Furthermore, IL-12 challenge of IFN-$\gamma^{+/+}$ but not IFN-$\gamma^{-/-}$ mice results in enhanced expression of PD-L1 in blood vessels of various tissues (M. Eppihimer, J. Leonard, personal communication). Thus in vivo, IFN-γ upregulation of PD-L1 expression on endothelial cells may play a significant role in attenuation of lymphocyte function at peripheral sites. Consistent with these findings, several studies have suggested an immunosuppressive role for IFN-γ. IFN-γ receptor–deficient mice develop accelerated collagen-induced arthritis (75), and IFN-γ blockade enhances EAE (76). In addition, IFN-γ treatment has been reported to confer resistance to EAE (77). Thus, the beneficial effect of IFN-γ reported in some autoimmune settings could be partly attributed to induction of PD-L1 ligand expression at sites of inflammation and subsequent downregulation of immune responses by PD-1 engagement.

Finally, PD-L1 and PD-L2 transcripts have been detected in various tumor cell lines (72). Additionally, cell-surface expression of PD-L1 has been reported in human breast cancer cell lines (72). These observations have led to the suggestion that tumors may escape immunosurveillance by attenuation of T cell responses upon PD-1 engagement. This hypothesis has implications for the development of new strategies for tumor immunotherapy, as one would predict that blockade of PD-1/PD-L interactions could enhance tumor-specific T cell responses.

ATTENUATION OF IMMUNE RESPONSES BY THE PD-1/PD-L PATHWAY

Identification of the PD-1 ligands, PD-L1 (B7-H1) and PD-L2 (B7-DC), and assessment of their interaction with PD-1 confirmed the negative regulatory function of PD-1 in immune responses (71, 72). Neither PD-L1 nor PD-L2 bound to CD28,

CTLA-4, or ICOS. Reciprocally, soluble forms of B7-1 and B7-2 did not bind PD-1. Experiments using T cells from PD-1-deficient mice formally demonstrated the functional consequences of PD-1/PD-L1 interactions (71). Proliferation of wild-type, but not PD-1-deficient, T cells was inhibited in the presence of PD-L1. Similarly, proliferation of human T cells was decreased in the presence of PD-L1. Cross-linking of PD-1 by PD-L2 upon TCR activation also resulted in decreased proliferation (72). Furthermore, PD-1 cross-linking by either ligand resulted in decreased IFN-γ, IL-10, IL-4, and IL-2 secretion (71, 72). These results indicate that, upon TCR activation, cross-linking of PD-1 by PD-L1 or PD-L2 leads to diminished immune responses. Thus, PD-1 cross-linking by either PD-L1 or PD-L2 results in similar outcomes, suggesting that these two ligands may have overlapping functions in vivo. However, additional information regarding their affinities for PD-1, as well as the generation of PD-L1- and PD-L2-deficient mice will be necessary to discern their potential roles in vivo.

Consistent with an inhibitory function, studies on PD-1^+ B cell lymphomas have shown that PD-1/BCR co-engagement results in inhibition of Ca^{2+} influx as well as hypophosphorylation of BCR downstream signaling molecules syk, phosphatidyl inositol-3, phospholipase C, and vav (70). SHP-1 and SHP-2, Src-homology-2 (SH-2) domain containing phosphatases, have been implicated in inhibitory signals mediated by NK receptors, with recruitment of both phosphatases upon tyrosine phosphorylation of ITIM motifs (64). A role for SHP-2 in CTLA-4 inhibitory signals has also been reported (78). Co-ligation of the BCR and PD-1 resulted in increased phosphorylation of SHP-2 and recruitment of SHP-2 to the PD-1 receptor (70). Interestingly, the carboxy-terminal tyrosine (TEYATI), and not the tyrosine within the canonical ITIM, has been implicated as necessary for SHP-2 binding (70). Similarly, TCR/PD-1 co-ligation resulted in increased phosphorylation of SHP-2 (72). Thus, these studies point to SHP-2 as a likely candidate involved in transducing inhibitory signals initiated by PD-1.

PD-1 appears to function as an attenuator of T cell responses, and the process by which this regulation occurs is distinct from activation-induced cell death (72). PD-1 engagement by either PD-L1 or PD-L2 results in cell cycle arrest. Activation of cells in the presence of either ligand leads to an accumulation of cells at the G0/G1 phase of the cell cycle. IL-2 production is drastically inhibited upon PD-1 engagement, while exogenous IL-2 can rescue PD-1-mediated cell cycle arrest (L. Carter, B. Carreno, unpublished observations). These observations suggest that PD-1 may affect T cell activation and proliferation by regulating IL-2 transcription. These data parallel those reported for CTLA-4, which most likely inhibits T cell cycle entry by regulating IL-2 transcription and mRNA stability (79–81).

The interplay between PD-1 and CD28 on T cell activation has also been examined. Optimal, but not suboptimal, CD28 costimulation can rescue PD-1-mediated inhibition (71). At low antigen concentrations, PD-1 signals can antagonize costimulation mediated by CD28. At high antigen levels, CD28 costimulation overrides the PD-1 inhibitory effect. Interestingly, PD-1 cell surface expression is highest

at low antigen concentration. Thus, a correlation can be established between antigen concentrations required for highest PD-1 expression and conditions in which downregulation of T cell responses are more readily observed (72).

Interestingly, two separate groups have concluded that PD-L1 (B7-H1) and PD-L2 (BC-DC) can function to costimulate T cell responses (73, 74). Both groups have reported that activation of T cells in the presence of suboptimal TCR signals, and either PD-L1 or PD-L2 results in increased proliferation. Additionally, anti-CD3 plus PD-L1.Fc activation results in increased secretion of IL-10, IFN-γ and GM-CSF but not IL-2 or IL-4 (73). Activation of T cells with anti-CD3 and PD-L2.Fc increases IFN-γ but not IL-4 or IL-10 (74). The discrepancy between these results and those of Freeman and colleagues (71, 72) raises the possibility that there may be additional receptors for PD-L1 and PD-L2. If, indeed, a second receptor with costimulatory function exists for PD-L ligands, this pathway would have symmetry with that of CD28/CTLA-4/B7. As speculated for CD28/CTLA-4, temporal regulation of receptor expression and affinities of ligands would then determine whether the costimulatory or inhibitory signals prevail.

PD-1 DEFICIENCY LEADS TO AUTOIMMUNE DISORDERS AND BREAKDOWN OF PERIPHERAL TOLERANCE

Consistent with its negative regulatory function, PD-1 deficiency in vivo results in the development of autoimmune disorders (69, 82, 83). C57BL/6- PD-$1^{-/-}$ mice consistently displayed splenomegaly, increased numbers of B lymphocytes and myeloid cells, and increased serum IgG2b, IgG3, and IgA (82). Antibody responses to T-independent, but not T-dependent, antigens were greatly enhanced in PD-$1^{-/-}$ mice relative to control littermates. Furthermore, PD-$1^{-/-}$ B cells displayed enhanced proliferation in response to BCR cross-linking. These mice spontaneously developed a lupus-like disease with age (69). At 6 months, PD-1-deficient mice displayed elevated serum IgG3 levels and increased IgG3 and C3 deposition in the glomeruli. At 14 months, approximately 50% of these mice had lupus-like glomerulonephritis and histological evidence of arthritis as well as granulomatous inflammation. Introduction of the lpr mutation (B6-lpr/lpr-PD-$1^{-/-}$) accelerated the onset and severity of disease. Thus, PD-1 deficiency in the C57BL/6 background resulted in the development of a late onset, chronic, progressive, lupus-like glomerulonephritis and arthritis, and the severity of disease was exacerbated by the absence of FAS-mediated apoptosis. Interestingly, introduction of the PD-1 deficiency into the Balb/c background resulted in a distinct autoimmune phenotype, with cardiomegaly, diffuse IgG1 deposition in cardiomyocytes, and high circulating levels of heart-tissue reactive IgG1 (83). This disease developed rapidly, and Balb/c-PD-$1^{-/-}$ mice died as early as 5 weeks of age. By 30 weeks, two thirds of mice had succumbed to disease. In contrast, no disease was observed in Balb/c-PD-$1^{-/-}$-RAG$^{-/-}$ mice, indicating that T and B cells are required for disease development. The distinct severity and phenotype of disease observed in

these two strains indicated that other genetic modifier genes in addition to the PD-1 deficiency contribute to the pathologies observed. However, in both genetic backgrounds, PD-1 deficiency appeared to alter the balance between activating and inhibitory signals, resulting in a loss of peripheral tolerance.

Further support for a role for PD-1 in the maintenance and/or induction of peripheral tolerance has come from studies examining responses to self-antigens (69). PD-1 deficient 2C TCR transgenic mice bred to the autoreactive background ($H\text{-}2^{b/d}$) exhibited growth retardation, splenomegaly, and lethal graft-vs-host disease. Massive infiltration of inflammatory cells in liver, heart, and lung was observed. Additionally, an increase in the total number of cells, most notably activated $CD8^+$ T cells, in the spleens was also reported. As PD-1 has a minimal, if any, role in negative selection in the thymus (69), these results point to PD-1 as negative regulator of self-reactivity in the periphery. Altogether, the outcome is a breakdown of peripheral tolerance to selected tissue antigens.

In vitro, PD-1 and CTLA-4 functions are quite similar. Engagement of either PD-1 or CTLA-4 results in inhibition of T cell proliferation, cytokine production, and cell cycle progression (3, 71). In vivo, deficiency in these molecules results in development of lymphoproliferative disorders, albeit with different degrees of severity. CTLA-4-deficient mice display very aggressive lymphoproliferative disorders and die at 21–28 days of age (6, 7, 84). Lymphocytic infiltration is observed in multiple organs. The disorder is characterized by a high frequency of T cell blasts (CD25, CD69, $CD44^{hi}$, $CD45RO^{low}$), with signs of T cell activation detected as early as 5–6 days after birth. In contrast, a less aggressive disorder is observed in PD-1-deficient mice (69, 83). Of the phenotypes reported, the most aggressive is the cardiomyopathy in Balb/c-PD-$1^{-/-}$ mice (83).

As deficiencies in either of these receptors result in breakdown of peripheral tolerance, both receptors must have critical and nonredundant functions in the maintenance of tolerance. These pathways may control T cell responses at two distinct points, with the first at the time of T cell activation in lymphoid tissues, and the second upon reactivation in peripheral sites. CTLA-4 would have a predominant role in regulating the threshold for T cell activation in lymphoid sites, where B7-1 and B7-2 are primarily expressed. Because both PD-1 and PD-1 ligands have broader expression patterns, PD-1 could be important in regulating thresholds of activation for T and B cells in both lymphoid and peripheral sites during inflammation. By controlling the magnitude of T cell responses at initiation and again at reactivation, these pathways could function as independent checkpoints to safeguard against self-reactivity.

B7-H3: A NEW B7-LIKE LIGAND

Human B7-H3 was recently identified as a new costimulatory member of the B7 family (85). B7-H3 shares from 26% to 33% amino acid identity with other members of the B7 family (Table 2). Northern analysis indicated that this gene is broadly expressed, with mRNA detected in most organs, as well as in immune tissues

including spleen, lymph node, thymus, bone marrow, and fetal liver. Tumor cell lines derived from nonlymphoid tissues were also positive for B7-H3 mRNA, but Molt-4, a lymphoblastic leukemia, and Raji, a Burkitt's lymphoma line, were not. B7-H3 expression was not detected in unstimulated PBL but could be induced in lymphoid cells by activation. B7-H3 protein was detected on GM-CSF-stimulated monocytes and IFN-γ activated dendritic cells, as well as on $CD3^+$ T cells activated with PMA and ionomycin.

B7-H3-transfected 293 cells did not bind CTLA-4.Fc, ICOS.Fc, or PD-1.Fc, which suggests that this B7 member binds a distinct receptor (85). Binding studies with the B7-H3.Fc protein further suggest that this receptor is present on activated T cells. Activation of human T cells with plate-bound anti-CD3 plus increasing concentrations of B7-H3.Fc resulted in a dose-dependent enhancement of proliferation relative to cells activated with anti-CD3 plus control Ig. B7-H3 enhancement of proliferative responses was not as profound as that observed with B7-1.Fc. B7-H3.Fc enhances proliferative responses of both $CD4^+$ and $CD8^+$ T cells. In addition, B7-H3.Fc stimulation increased secretion of IFNγ in 50% of cell donors tested. Interestingly, expression of B7-H3 in a melanoma line resulted in increased lytic activity of melanoma-specific T cells. The receptor for this newest member of the B7 family remains to be identified, and further elucidation of B7-H3 function in immune responses is needed.

NEW MEMBERS OF THE B7 AND CD28 FAMILY?

The recent expansion of the B7 family and its receptors raises the question of whether there are additional members of this family with immune function. Certainly, the receptor for B7-H3 remains to be identified. Conflicting results indicating that PD-L1 and PD-L2 can both costimulate and inhibit immune responses raise the possibility of additional receptors for these ligands. In addition, experiments demonstrating B7-dependent responses in mice deficient for CD28 and CTLA-4 suggest that there are additional receptors for the B7 molecules (86, 87). Finally, experiments evaluating ICOS-L often use the ICOS.Fc reagent to define this ligand. Antibody reagents for ICOS-L are needed to confirm that these interactions do not include contributions of additional ligands for ICOS. In addition to the B7-like ligands and their receptors described above, molecules with homologies to these families have been described. In each of these cases, data are too limited to currently include these molecules as bona fide members of the B7-family and their receptors. A key question is whether these related proteins have a role in immune responses.

Members of the butyrophilin gene family share homology with the B7-family of immunoregulatory receptors (88, 89). Butyrophilin is a 66-kDa type I transmembrane protein that forms a major component of milk fat globule membrane (90, 91). It has no reported role in the immune system. Six additional family members were identifed by homology and genomic location and form two subfamilies (92, 93). The seven members of the butyrophilin gene family are located in a cluster

on human chromosome 6p22.1, telomeric to the class I MHC (89, 93). Like the B7 proteins, butyrophilins are type I transmembrane proteins with IgV and IgC extracellular domains. However, they also have unique cytoplasmic domains, including a series of heptad repeats and a B30.2 domain, which may mediate protein interactions (89, 93). Butyrophilin genes are expressed at low levels in most tissues (93). Interestingly, the predominant transcripts of the BTN3 subfamily do not express the B30.2 domain due to alternate splicing (93), making the BTN3 subfamily proteins more similar to the B7 gene family.

Linsley et al. (88) also noted that the myelin protein MOG has homology with B7-1, B7-2, and butyrophilin, although MOG contains only an IgV extracellular domain. MOG is an autoantigen in EAE, and immunization of susceptible rats with the IgV domain of butyrophilin also results in an inflammatory response in the CNS due to cross-reactivity of T cells to MOG-derived epitopes (94). MOG maps to 6p21 in humans, centromeric to the butyrophilin genes and telomeric from the class I and II gene clusters (95, 96). Although MOG can act as an autoantigen in animal models of multiple sclerosis, as yet there are no data defining an interacting protein for MOG, nor any data implicating MOG as a costimulatory protein in immune function.

Beyond these defined family members, other potential homologous sequences have been identified in database searches (97). These include SIRPα and β, transmembrane proteins expressed in the immune system; HHLA2, an endogenous retroviral sequence; MCAM, a melanoma adhesion protein; and VEJAM, vascular endothelial junction–associated molecule, as well as a few novel sequences with similar levels of homology. Additionally, Linsley et al. (88) noted the homology of the chicken B-G gene to the B7 family.

Homology searches to the CD28 receptor family can also be carried out, and there is sufficient homology between CD28, ICOS, and CTLA-4 to detect these as related sequences. However, PD-1 is less homologous to this family, suggesting that the receptors for the B7 family of ligands may be less conserved. Indeed, as each of these receptors contains only a single extracellular IgV domain, many proteins of the Ig-superfamily can be detected with weak homology, including TCRα and Igκ proteins. This suggests that functional tests of members of the Ig superfamily will be critical in identifying new members of costimulatory and inhibitory receptors and ligands related to CD28 and B7.

PERSPECTIVES

The sequencing of the human genome has led to an explosion of gene identification, along with information about gene clusters. The new challenge is to ascribe function to new genes with sequence relationships to known genes. For the family of genes related to the CD28 and B7 molecules, this has led to an appreciation that these receptor-ligand interactions will include both costimulatory and inhibitory receptors, and that multiple receptor-ligand interactions are possible. In addition, regulation of the immune response can be affected by signals delivered through

multiple costimulatory or inhibitory receptors, each of which is simultaneously expressed by the interacting cells. Integration of these various signals with signals through antigen-specific receptors then determines the outcome of that cellular interaction. Regulation of immune responses in both the priming events in immune sites and in activation or attenuation in peripheral sites can occur through these pathways. An understanding of which interactions are critical at various steps in immune responses will allow intervention in immune-mediated diseases through precise manipulation of these pathways.

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